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(54) Title: APO-3 LIGAND

		A	B	C	D	E
Apo3L	99	HETRARARALAAHAYEVHPRP-GQ-	-DGAQAGVGDGTSGWEEARIN-	--SSSPRLRYNRQIG		
TNF	82	R-TPSDK-PVAHVANPQAEGQ-	--LQ-	--WLNRRAAHL-LLAGVLER-DN		
CD40L	114	QKGDONPOYAAHVSEARS-	--KTTSVLQ-	--WAEGKYYT-MSNNVLTELNG		
Ltb	80	ETDLSPGLPMVHGGAPLK-GQ-	--GLG-	--WETTKEOA-WLTSQTQFSDAE		
Apo2L	115	KERGPQH-VASRHTGTRGRSNTLSSPNNSNEKAAGRKINSMESSRSRGHSFLSNLH-LR-NG				
CD95L	138	PEKKELD-KVAVH-TGKSNS-	--RSMPL-	--WEDTYGIV-LLSG-VKYK-KG		
Lta	56	LAHSTIKK-PVAKHLDGDPSK-	--QNSLL-	--WRANTDRA-FLODGFSLS-NN		
				F	G	H
Apo3L	152	HIVTRA-QVLLVLTQVTHDE-G-	--KAVYIYLKDLLVDGV-	--LAIRCDBEPSATA		
TNF	123	QVAVTSESSKLVLIKSVVLYKQGCCPS-	--THVLLIHTISRIAVST-	--OTKVMDLSSAIKSP		
CD40L	144	QSTWVKROQKQVWVIAQVTCNSNREABSQAPPIASLCLKSPG-	--RPERILLRAANTH			
Ltb	123	QHALFQDQHHLVHACLVGTVBRAPPGGGDPQGRSVTLRSSLITRAGGAYGRTPELLEGAET				
Apo2L	173	SHVILHKHDFKFLD2HOTSFHCPBEIKEKENDOKQMVQVITYTIS-X-	--FD-PILLMSARN			
CD95L	180	GHVIVMHNQHNVVAVVYENRSG-	--EHLFESHHVQVMMRN8KYP-	--QDIDVMMEGKOMSY		
Lta	98	SILVWTSGIYWVQDQVVSOKAYSPKATSSPLVTAHEVOLPSQX-	--RHVHPVLL8SQKMV			

### (57) Abstract

A tumor necrosis factor and lymphotxin homolog having apoptotic activity, identified as Apo-3 Ligand, is provided. Nucleic acid molecules encoding Apo-3 ligand, chimeric molecules and antibodies to Apo-3 Ligand are also provided.

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## Apo-3 Ligand

FIELD OF THE INVENTION

The present invention relates generally to the identification and isolation of novel DNA and to the recombinant production of novel polypeptides, designated herein as "Apo-3 Ligand".

BACKGROUND OF THE INVENTION

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as myc, rel, and E1A, and tumor suppressors, like p53, have been

reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin- $\alpha$ "), lymphotoxin- $\beta$  ("LT- $\beta$ "), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); Wiley et al., Immunity, 3:673-682 (1995); Browning et al., Cell, 72:847-856 (1993); Armitage et al. Nature, 357:80-82 (1992)]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc.

Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., 5 Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface 10 receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. More recently, the cloning of recombinant 15 soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting 20 from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., *supra*; Loetscher et al., *supra*; Smith et al., *supra*; Nophar et al., *supra*; Kohno et al., *supra*]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- 25 amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 30 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., *supra*.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor 35 receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., *supra* and Itoh et al., Cell, 66:233-243 (1991)]. CRDs are also found in the 40 soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al.,

Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, most receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. Such newly identified members of the TNFR family include CAR1, HVEM and osteoprotegerin (OPG) [Brojatsch et al., Cell, 87:845-855 (1996); Montgomery et al., Cell, 87:427-436 (1996); Marsters et al., J. Biol. Chem., 272:14029-14032 (1997); Simonet et al., Cell,

89:309-319 (1997)]. Unlike other known TNFR-like molecules, Simonet et al., *supra*, report that OPG contains no hydrophobic transmembrane-spanning sequence.

5 In Marsters et al., *Curr. Biol.*, 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., *Curr. Biol.*, 6:1669 (1996)]. Apo-3 has also 10 been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., *Science*, 274:990 (1996); Kitson et al., *Nature*, 384:372 (1996); Bodmer et al., *Immunity*, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., *Science*, 276:111-113 15 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

In Sheridan et al., *Science*, 277:818-821 (1997) and Pan 20 et al., *Science*, 277:815-818 (1997), another molecule believed to be a receptor for the Apo-2 ligand (TRAIL) is described. That molecule is referred to as DR5 (it has also been alternatively referred to as Apo-2). Like DR4, DR5 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis.

25 In Sheridan et al., *supra*, a receptor called DcR1 (or alternatively, Apo-2DcR) is disclosed as being a potential decoy receptor for Apo-2 ligand (TRAIL). Sheridan et al. report that DcR1 can inhibit Apo-2 ligand function *in vitro*. See also, Pan et al., *supra*, for disclosure on the decoy receptor referred to as TRID.

30 For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, *supra*.

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by 35 three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, *Science*, 267:1445 (1995); Chinnaiyan et al., *Science*, 275:1122-1126 (1997); Wang et al., *Cell*, 90:1-20 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apol (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, *Current Biology*, 6:555-562 (1996); Fraser and Evan, *Cell*; 40 *85*:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., *Cell*, 74:845-853 (1993); Hsu et al., *Cell*, 84:299-308 (1996)]. In addition to some

ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., *supra*; Nagata, *Cell*, **88**:355 (1997)]. Death domains are also found in several metazoan proteins  
5 that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, *Cell*, **81**:479-482 (1995)]. Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95  
10 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., *Cell*, **81**:505-512 (1995); Boldin et al., *J. Biol. Chem.*, **270**:387-391 (1995); Hsu et al., *supra*; Chinnaiyan et al., *J. Biol. Chem.*, **271**:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-  
15 related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., *Cell*, **85**:803-815 (1996); Muzio et al., *Cell*, **85**:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some  
20 critical aspects of the cell death programme [Fraser and Evan, *supra*].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the  
25 mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., *Cell*, **69**:597-604 (1992); Tewari et al., *Cell*, **81**:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., *Nature*, **375**:78-81 (1995); Tewari et al., *J. Biol. Chem.*, **270**:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., *Curr. Op. Genet. Develop.*, **6**:39-44 (1996)]. NF- $\kappa$ B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., *Genes Develop.*, **9**:2723-2735 (1996); Baldwin, *Ann. Rev. Immunol.*, **14**:649-681 (1996)]. In its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor  
40 family; upon inactivation of the I $\kappa$ B in response to certain stimuli,

released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

5

SUMMARY OF THE INVENTION

Applicants have identified a cDNA clone that encodes a novel polypeptide, designated in the present application as "Apo-3 Ligand." The Apo-3 ligand of the invention is the same molecule previously referred to by Applicants as "DNA30879."

10

In one embodiment, the invention provides an isolated nucleic acid molecule comprising DNA encoding Apo-3 Ligand polypeptide. Optionally, the isolated nucleic acid comprises DNA encoding Apo-3 Ligand polypeptide having amino acid residues 47 to 249 or 1 to 249 of Fig. 1 (SEQ ID NO:1), or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions.

15

The isolated nucleic acid may comprise the Apo-3 Ligand cDNA insert of the vector deposited as ATCC 209358 which includes the nucleotide sequence encoding Apo-3 Ligand.

20

In another embodiment, the invention provides a vector comprising DNA encoding Apo-3 Ligand polypeptide. A host cell comprising such a vector is also provided. By way of example, the host cells may be CHO cells, *E. coli*, or yeast. A process for producing Apo-3 Ligand polypeptides is further provided and comprises culturing host cells under conditions suitable for expression of Apo-3 Ligand and recovering Apo-3 Ligand from the cell culture.

25

In another embodiment, the invention provides isolated Apo-3 Ligand polypeptide. In particular, the invention provides isolated native sequence Apo-3 Ligand polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 47 to 249 or residues 1 to 249 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-3 Ligand polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209358.

30

In another embodiment, the invention provides chimeric molecules comprising Apo-3 Ligand polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-3 Ligand fused to an epitope tag sequence or a Fc region of an immunoglobulin.

35

In another embodiment, the invention provides an antibody which specifically binds to Apo-3 Ligand polypeptide. Optionally, the antibody is a monoclonal antibody.

In another embodiment, the invention provides methods of using Apo-3 Ligand polypeptide. Included in such methods are methods of inducing apoptosis in mammalian cells using Apo-3 Ligand polypeptide.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence (SEQ ID NO:2) of a cDNA for human Apo-3 Ligand and its derived amino acid sequence (SEQ ID NO:1).

10       Figure 2 shows an EST sequence (SEQ ID NO:3) discussed in Example 1.

Figure 3A shows an alignment and comparison of the full length sequences of Apo-3 Ligand polypeptide (Apo-3L), human lymphotoxin-beta (hLTb) and human CD40 ligand (hCD40L).

15       Figure 3B shows an alignment and comparison of the C-terminal sequences of Apo-3 Ligand polypeptide (Apo3L), TNF-alpha (TNF), hCD40L, hLTb, Apo-2L, CD95L and LT-alpha (LTa). Residues conserved in two or more ligands are shaded. Regions that form beta-strands in the crystal structures of TNF-alpha and LT-alpha are marked by overhead lines.

20       Figure 4 shows expression of Apo-3 Ligand mRNA in human fetal and adult tissues and cell lines as analyzed by Northern hybridization.

25       Figure 5 shows NF- $\kappa$ B activation by Apo-3L in HeLa cells (5A) and 293 cells (5B and 5C).

Figure 6 is a bar graph showing apoptotic activity in HeLa cells transfected with Apo-3 Ligand. The increased apoptotic activity was blocked when the cells were incubated with the caspase inhibitor, z-VAD-fmk.

30       Figure 7 shows transfection of 293 cells with Apo-3 Ligand resulted in increased JNK/SAPK activity.

Figure 8A shows a Western blot of co-immunoprecipitated ligands/receptors. The data shows that Apo-3L specifically binds to Apo-3.

35       Figure 8B is a bar graph showing apoptotic activity of Apo-3L and specific complex formation between Apo-3L and Apo-3.

Figure 9A shows Apo-3L induced apoptosis in MCF-7 cells (pre-treated with CHX) as measured by phase and fluorescence microscopy.

40       Figure 9B shows the apoptotic effect of combined treatment of MCF-7 cells with Apo-3L and CHX.

Figure 9C shows DNA fragmentation analysis of HeLa S3 treated with Apo-3L or Apo-3L plus DEVD-fmk or zVAD-fmk.

Figure 9D shows the results of transfection of MCF-7 cells with a caspase inhibitor (p35 or CrmA) or FADD mutant followed by incubation with CHX and Apo-3L. The assay shows the transfection with a dominant-negative mutant of FADD prevented apoptosis induction by Apo-3L.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

10 I. Definitions

The terms "Apo-3 Ligand polypeptide", "Apo-3 Ligand", and "Apo-3L" when used herein encompass native sequence Apo-3 Ligand and Apo-3 Ligand variants (which are further defined herein). The Apo-3 Ligand may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-3 Ligand" comprises a polypeptide having the same amino acid sequence as an Apo-3 Ligand derived from nature. Such native sequence Apo-3 Ligand can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-3 Ligand" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-3 Ligand (e.g., soluble forms containing for instance, an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-3 Ligand. In one embodiment of the invention, the native sequence Apo-3 Ligand is a mature or full-length native sequence Apo-3 Ligand polypeptide comprising amino acids 1 to 249 of Fig. 1 (SEQ ID NO:1).

Alternatively, the Apo-3 Ligand polypeptide comprises amino acids 47 to 249 of Fig. 1 (SEQ ID NO:1). Optionally, the Apo-3 Ligand polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209358.

The "Apo-3 Ligand extracellular domain" or "Apo-3 Ligand ECD" refers to a form of Apo-3 Ligand which is essentially free of the transmembrane and cytoplasmic domains of Apo-3 Ligand. Ordinarily, Apo-3 Ligand ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-3 Ligand ECD will comprise amino acid residues 47 to 249 of Fig. 1 (SEQ ID NO:1). It will be understood by the skilled artisan that the transmembrane domain identified for the Apo-3 Ligand polypeptide of the present invention is identified pursuant to criteria routinely employed in

the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain specifically mentioned herein. Accordingly, the Apo-3 Ligand ECD may 5 optionally comprise amino acids X to 249 of Figure 1 (SEQ ID NO:1) wherein X is any one of amino acid residues 42 to 52 of Figure 1 (SEQ ID NO:1).

"Apo-3 Ligand variant" means an active Apo-3 Ligand as defined below having at least about 80% amino acid sequence identity 10 with the Apo-3 Ligand having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence Apo-3 Ligand or with an Apo-3 Ligand ECD sequence. Such Apo-3 Ligand variants include, for instance, Apo-3 Ligand polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of 15 the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-3 Ligand variant will have at least about 80% or 85% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID 20 NO:1).

"Percent (%) amino acid sequence identity" with respect to the Apo-3 Ligand sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-3 Ligand sequence, 25 after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in 30 the art, for instance, using publicly available computer software such as BLAST, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

35 "Percent (%) nucleic acid sequence identity" with respect to the Apo-3 Ligand sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the Apo-3 Ligand sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum 40 percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using

publicly available computer software such as BLAST, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length  
5 of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-3 Ligand, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can  
10 be made, or which can be identified by some other agent, yet is short enough such that it does not interfere with activity of the Apo-3 Ligand. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes.

Suitable tag polypeptides generally have at least six amino acid  
15 residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its  
20 natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified  
25 (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within  
30 recombinant cells, since at least one component of the Apo-3 Ligand natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-3 Ligand nucleic acid molecule is a  
35 nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-3 Ligand nucleic acid. An isolated Apo-3 Ligand nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-3  
40 Ligand nucleic acid molecules therefore are distinguished from the Apo-3 Ligand nucleic acid molecule as it exists in natural cells. However, an isolated Apo-3 Ligand nucleic acid molecule includes Apo-

3 Ligand nucleic acid molecules contained in cells that ordinarily express Apo-3 Ligand where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences  
5 necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

10 Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or  
15 enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a  
20 secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

25 The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-3 Ligand monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-3 Ligand antibody compositions with polyepitopic specificity. The term "monoclonal antibody" as used herein refers to  
30 an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts.

"Biologically active" and "desired biological activity"  
35 for the purposes herein mean having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

40 The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm,

loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which 5 are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The terms "cancer" and "cancerous" refer to or describe 10 the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, 15 blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, neuroblastoma, cervical cancer, ovarian cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial cancer, salivary gland cancer, kidney cancer, prostate cancer, vulval cancer, thyroid 20 cancer, hepatic carcinoma, and various types of head and neck cancer.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

25

## II. Compositions and Methods of the Invention

### A. Full-length Apo-3 Ligand

The present invention provides newly identified and isolated nucleotide sequences encoding polypeptides referred to in 30 the present application as Apo-3 Ligand. In particular, Applicants have identified and isolated cDNA encoding an Apo-3 Ligand polypeptide, as disclosed in further detail in the Examples below. Using BLAST and FastA sequence alignment computer programs, Applicants found that a full-length native sequence Apo-3 Ligand 35 (shown in Figure 1 and SEQ ID NO:1) has 23.4% amino acid sequence identity with human lymphotoxin-beta, and 19.8% amino acid sequence identity with CD40 ligand, and significant but lower identity to other members of the TNF cytokine family. As shown in the Examples below, Apo-3 Ligand polypeptide (full length and soluble forms) was 40 found to have apoptotic activity.

B. Apo-3 Ligand Variants

In addition to the full-length native sequence Apo-3 Ligand and soluble ECD forms described herein, it is contemplated that Apo-3 Ligand variants can be prepared. Apo-3 Ligand variants  
5 can be prepared by introducing appropriate nucleotide changes into the Apo-3 Ligand nucleotide sequence, or by synthesis of the desired Apo-3 Ligand polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-3 Ligand, such as changing the number or position of  
10 glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-3 Ligand or in various domains of the Apo-3 Ligand described herein, can be made, for example, using any of the techniques and guidelines  
15 for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-3 Ligand that results in a change in the amino acid sequence of the Apo-3 Ligand as compared with the native sequence Apo-3  
20 Ligand. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-3 Ligand. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the  
25 Apo-3 Ligand with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a  
30 leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity  
35 in any of the *in vitro* assays described in the Examples below.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known

techniques can be performed on the cloned DNA to produce the Apo-3 Ligand variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among 5 the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the 10 variant. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric 15 amino acid can be used.

#### C. Modifications of Apo-3 Ligand

Covalent modifications of Apo-3 Ligand are included within the scope of this invention. One type of covalent 20 modification includes reacting targeted amino acid residues of the Apo-3 Ligand with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-3 Ligand. Derivatization with bifunctional agents is useful, for instance, for crosslinking Apo-3 Ligand to a water-insoluble support matrix or surface for use in the method for 25 purifying anti-Apo-3 Ligand antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)-dithio]propioimidate.

Other modifications include deamidation of glutaminyl and 35 asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal 40 carboxyl group.

Another type of covalent modification of the Apo-3 Ligand polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide.

"Altering the native glycosylation pattern" is intended for purposes 5 herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-3 Ligand, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-3 Ligand.

Addition of glycosylation sites to the Apo-3 Ligand polypeptide may be accomplished by altering the amino acid sequence. 10 The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-3 Ligand (for O-linked glycosylation sites). The Apo-3 Ligand amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding 15 the Apo-3 Ligand polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the Apo-3 Ligand polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are 20 described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-3 Ligand polypeptide may be accomplished chemically or enzymatically or 25 by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of 30 carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of Apo-3 Ligand comprises linking the Apo-3 Ligand polypeptide to one of a variety of 35 nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The Apo-3 Ligand of the present invention may also be 40 modified in a way to form a chimeric molecule comprising Apo-3 Ligand fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of the

Apo-3 Ligand with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-3 Ligand. The presence of such epitope-tagged forms of the Apo-3

- 5 Ligand can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-3 Ligand to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a  
10 fusion of the Apo-3 Ligand with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule. In particular, the chimeric molecule may comprise a Apo-3 Ligand which includes amino acids 47 to 249 of Fig. 1 (SEQ ID NO:1) fused to  
15 a His-tag molecule.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol.,  
20 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology,  
5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp  
25 et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

30 The Apo-3 Ligand of the invention may also be modified in a way to form a chimeric molecule comprising Apo-3 Ligand fused to a leucine zipper. Various leucine zipper polypeptides have been described in the art. See, e.g., Landschulz et al., Science,  
240:1759 (1988); WO 94/10308; Hoppe et al., FEBS Letters, 344:1991  
35 (1994); Maniatis et al., Nature, 341:24 (1989). It is believed that use of a leucine zipper fused to Apo-3 Ligand may be desirable to assist in dimerizing or trimerizing soluble Apo-3 Ligand in solution. Those skilled in the art will appreciate that the leucine zipper may be fused at either the 5' or 3' end of the Apo-3 Ligand molecule.

D. Preparation of Apo-3 Ligand

The description below relates primarily to production of Apo-3 Ligand by culturing cells transformed or transfected with a vector containing Apo-3 Ligand nucleic acid. It is, of course, 5 contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-3 Ligand. For instance, the Apo-3 Ligand sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, e.g., Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, 10 CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)]. In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the 15 Apo-3 Ligand may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the full-length Apo-3 Ligand.

1. Isolation of DNA Encoding Apo-3 Ligand

DNA encoding Apo-3 Ligand may be obtained from a cDNA 20 library prepared from tissue believed to possess the Apo-3 Ligand mRNA and to express it at a detectable level. Accordingly, human Apo-3 Ligand DNA can be conveniently obtained from a cDNA library prepared from human tissue, such as described in the Examples. The Apo-3 Ligand-encoding gene may also be obtained from a genomic 25 library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-3 Ligand or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the 30 selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-3 Ligand is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 35 1995)].

The Examples below describe techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that 40 false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the

art, and include the use of radiolabels like  $^{32}\text{P}$ -labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

5 Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across  
10 the full-length sequence can be determined through sequence alignment using computer software programs such as ALIGN, DNASTAR, and INHERIT.

Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and,  
15 if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

## 2. Selection and Transformation of Host Cells

20 Host cells are transfected or transformed with expression or cloning vectors described herein for Apo-3 Ligand production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as  
25 media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., supra.

Methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment  
35 employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO  
40 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) can be employed. General

aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to 15 eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635).

In addition to prokaryotes, eukaryotic microbes such as 20 filamentous fungi or yeast are suitable cloning or expression hosts for Apo-3 Ligand-encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism.

Suitable host cells for the expression of glycosylated 25 Apo-3 Ligand are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila S2* and *Spodoptera Sf9*, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by 30 SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); human 35 lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

### 3. Selection and Use of a Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding 40 Apo-3 Ligand may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are

publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate 5 restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing 10 one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The Apo-3 Ligand may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide 15 having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-3 Ligand DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of 20 the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, 25 the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as 30 viral secretory leaders.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the 35 plasmid pBR322 is suitable for most Gram-negative bacteria, the  $2\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a 40 selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or

tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-3 Ligand nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

Expression and cloning vectors usually contain a promoter operably linked to the Apo-3 Ligand nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (*trp*) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-3 Ligand.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytchrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose

utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

Apo-3 Ligand transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the 5 genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous 10 mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding the Apo-3 Ligand by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually 15 about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell 20 virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-3 Ligand coding sequence, but is preferably located at a site 5' from the promoter.

25 Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', 30 untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-3 Ligand.

Still other methods, vectors, and host cells suitable for 35 adaptation to the synthesis of Apo-3 Ligand in recombinant vertebrate cell culture are described in Gething et al., *Nature*, 293:620-625 (1981); Mantei et al., *Nature*, 281:40-46 (1979); EP 117,060; and EP 117,058.

#### 4. Detecting Gene Amplification/Expression

40 Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas,

Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, 5 including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

10 Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids

15 may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-3 Ligand polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-3 Ligand DNA and encoding a specific

20 antibody epitope.

##### 5. Purification of Polypeptide

Forms of Apo-3 Ligand may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. 25 Triton-X 100) or by enzymatic cleavage. Cells employed in expression of Apo-3 Ligand can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desired to purify Apo-3 Ligand from recombinant 30 cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel 35 filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the Apo-3 Ligand. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification:Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) 40 selected will depend, for example, on the nature of the production

process used and the particular Apo-3 Ligand produced.

E. Uses for Apo-3 Ligand

Nucleotide sequences (or their complement) encoding Apo-3

5 Ligand have various applications in the art of molecular biology, including uses as hybridization probes, in chromosome and gene mapping and in the generation of anti-sense RNA and DNA. Apo-3 Ligand nucleic acid will also be useful for the preparation of Apo-3 Ligand polypeptides by the recombinant techniques described herein.

10 The full-length native sequence Apo-3 Ligand (Fig. 1; SEQ ID NO:2) gene, or portions thereof, may be used as hybridization probes for a cDNA library to isolate, for instance, still other genes (like those encoding naturally-occurring variants of Apo-3 Ligand or Apo-3 Ligand from other species) which have a desired sequence identity to the Apo-3 Ligand sequence disclosed in Fig. 1 (SEQ ID NO:2). Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from the nucleotide sequence of SEQ ID NO:2 or from genomic sequences including promoters, enhancer elements and introns of native sequence 15 Apo-3 Ligand. By way of example, a screening method will comprise isolating the coding region of the Apo-3 Ligand gene using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , or enzymatic labels such as 20 alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the Apo-3 Ligand gene of the present invention can be used to screen 25 libraries of human cDNA, genomic DNA or mRNA to determine which members of such libraries the probe hybridizes to. Hybridization techniques are described in further detail in the Examples below.

30 Nucleotide sequences encoding a Apo-3 Ligand can also be used to construct hybridization probes for mapping the gene which encodes that Apo-3 Ligand and for the genetic analysis of individuals with genetic disorders. The nucleotide sequences provided herein may 35 be mapped to a chromosome and specific regions of a chromosome using known techniques, such as *in situ* hybridization, linkage analysis against known chromosomal markers, and hybridization screening with libraries.

Screening assays can be designed to find lead compounds 40 that mimic the biological activity of a native Apo-3 Ligand or a ligand or receptor for Apo-3 Ligand. Such screening assays will include assays amenable to high-throughput screening of chemical

libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

Nucleic acids which encode Apo-3 Ligand or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-3 Ligand can be used to clone genomic DNA encoding Apo-3 Ligand in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-3 Ligand. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-3 Ligand transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-3 Ligand introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-3 Ligand. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Alternatively, non-human homologues of Apo-3 Ligand can be used to construct a Apo-3 Ligand "knock out" animal which has a defective or altered gene encoding Apo-3 Ligand as a result of homologous recombination between the endogenous gene encoding Apo-3 Ligand and altered genomic DNA encoding Apo-3 Ligand introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-3 Ligand can be used to clone genomic DNA encoding Apo-3 Ligand in accordance with established techniques. A portion of the genomic DNA

encoding Apo-3 Ligand can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector 5 [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 10 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable 15 pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be 20 characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-3 Ligand polypeptide.

As described herein, it was found that Apo-3 Ligand 25 induces apoptosis in various cancer cells. Accordingly, in one embodiment of the invention, there are provided methods of inducing apoptosis in mammalian cancer cells. Among these methods are methods of administering Apo-3 Ligand to mammals to treat cancer. Suitable carriers for Apo-3 Ligand, for instance, and their formulations, are 30 described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the carrier include buffers such as saline, Ringer's solution and 35 dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7.4 to about 7.8. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. 40 It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of the Apo-3 Ligand

molecule being administered.

Administration to a mammal may be accomplished by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure 5 delivery to the bloodstream in an effective form.

Effective dosages and schedules for administration may be determined empirically, and making such determinations is within the skill in the art.

In methods of treating cancer using the Apo-3 Ligand 10 described herein, it is contemplated that other, additional therapies may be administered to the mammal, and such includes but is not limited to, chemotherapy and radiation therapy, immunoadjuvants, cytokines, and antibody-based therapies. Examples include interleukins (e.g., IL-1, IL-2, IL-3, IL-6), leukemia inhibitory 15 factor, interferons, TGF-beta, erythropoietin, thrombopoietin, HER-2 antibody and anti-CD20 antibody. Other agents known to induce apoptosis in mammalian cells may also be employed, and such agents include TNF- $\alpha$ , TNF- $\beta$  (lymphotoxin- $\alpha$ ), CD30 ligand, and 4-1BB ligand.

Chemotherapies contemplated by the invention include 20 chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotapec, Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. Preparation and dosing schedules for such chemotherapy 25 may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Cancer Chemotherapy Service Ed.*, M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapy is preferably administered in a pharmaceutically- 30 acceptable carrier, such as those described above. The Apo-3 Ligand may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of Apo-3 Ligand and therapeutic agent depend, for example, on what type of drugs are used, the cancer being treated, and the scheduling and routes of 35 administration but would generally be less than if each were used individually.

Following administration of Apo-3 Ligand to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, 40 tumor mass may be observed physically or by standard x-ray imaging techniques.

**F. Anti-Apo-3 Ligand Antibodies**

The present invention further provides anti-Apo-3 Ligand antibodies. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

**1. Polyclonal Antibodies**

The Apo-3 Ligand antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-3 Ligand polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

25

**2. Monoclonal Antibodies**

The Apo-3 Ligand antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-3 Ligand polypeptide or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press,

(1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed.

The hybridoma cells may be cultured in a suitable culture medium  
5 that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and  
10 thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such  
15 as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. Human myeloma and mouse-human heteromyeloma cell lines also have been described for  
20 the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are  
25 cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-3 Ligand. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay  
30 (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the  
35 clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

40 The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for

example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567.

- 5   DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a
- 10   preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant
- 15   host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., *supra*] or by covalently joining to the immunoglobulin coding sequence all or part of the
- 20   coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

- 25   The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

- 30   In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

### 3. Humanized Antibodies

- 35   The Apo-3 Ligand antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab',

F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also 5 comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially 10 all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are 15 those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann 20 et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen 25 et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an 30 intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues 35 from analogous sites in rodent antibodies.

Human antibodies can also be produced using various 40 techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and

Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)).

5

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding 10 specificities is for the Apo-3 Ligand, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific 15 antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 20 ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

25 Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH<sub>2</sub>, and CH<sub>3</sub> regions. It is preferred to have the first heavy-chain constant region (CH<sub>1</sub>) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into 30 separate expression vectors, and are co-transfected into a suitable host organism. For further details of generating bispecific 35 antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

##### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, 5 been proposed to target immune system cells to unwanted cells [U.S. Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. 10 For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptoputyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

15

##### G. Uses for Apo-3 Ligand Antibodies

The Apo-3 Ligand antibodies of the invention have various utilities. For example, Apo-3 Ligand antibodies may be used in diagnostic assays for Apo-3 Ligand, e.g., detecting its expression in 20 specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) 25 pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 30 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

- Apo-3 Ligand antibodies also are useful for the affinity purification of Apo-3 Ligand from recombinant cell culture or natural sources. In this process, the antibodies against Apo-3 Ligand are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-3 Ligand to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-3 Ligand, which is bound to the immobilized antibody.
- 5 Finally, the support is washed with another suitable solvent that will release the Apo-3 Ligand from the antibody.
- 10

\*\*\*\*\*

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

20

#### EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the 25 following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-3 Ligand

An expressed sequence tag (EST) DNA database (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) was searched and an EST was identified which showed homology to human Apo-2 ligand. The EST sequence is shown in Figure 2 (SEQ ID NO:3). A human fetal kidney 35 cDNA library was then screened. mRNA isolated from human fetal kidney tissue (Clontech) was used to prepare the cDNA library. This RNA was used to generate an oligo dT primed cDNA library in the vector pRK5D using reagents and protocols from Life Technologies, Gaithersburg, MD (Super Script Plasmid System). In this procedure, 40 the double stranded cDNA was sized to greater than 1000 bp and the SalI/NotI linker cDNA was cloned into XhoI/NotI cleaved vector. pRK5D is a cloning vector that has an sp6 transcription initiation

site followed by an SfiI restriction enzyme site preceding the XhoI/NotI cDNA cloning sites. The library was screened by hybridization with a synthetic oligonucleotide probe:

CCAGGCCCTCTGCGCTACAACCGCCAGATCGGGGAGTTATAGTCACCCGG (SEQ ID NO:4)

5 based on the EST.

A cDNA clone was sequenced in entirety. A nucleotide sequence of Apo-3 Ligand is shown in Figure 1 (SEQ ID NO:2). Clone DNA30879-1152 contains a single open reading frame with an apparent translational initiation site at nucleotide positions 92-94 [Kozak et al., *supra*] (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 249 amino acids long and has a calculated mass of approximately 27 kDa. Hydropathy analysis suggests a type II transmembrane protein typology, with a putative cytoplasmic region (amino acids 1-19); transmembrane region (amino acids 20-46); and extracellular region (amino acids 47-249) (see Figure 1). A potential N-linked glycosylation site appears at amino acid position 139 in the sequence of SEQ ID NO:1. Clone DNA30879-1152 has been deposited with ATCC and is assigned ATCC deposit no. 209358. Apo-3 Ligand polypeptide is obtained or obtainable by expressing the molecule encoded by the cDNA insert of the deposited ATCC 209358 vector. Digestion of the vector with XbaI and NotI will yield two inserts: 446 bp and 959 bp.

Based on a BLAST and FastA sequence alignment analysis (using the ALIGN computer program) of the full-length sequence, Apo-3 Ligand shows amino acid sequence identity to several members of the TNF cytokine family, and particularly, to human lymphotoxin-beta (23.4%) and human CD40 ligand (19.8%) (see Figure 3A). In an alignment analysis of the C-terminal ECD sequence, Apo-3 Ligand shows the highest amino acid sequence identity to TNF-alpha (22.5%); certain amino acid sequence identity was also found to CD40L (21.2%), LT-beta (20.5%), Apo-2L (19.9%), LT-alpha (15.2%), and CD95L (13.9%) (see Figure 3B). Most of the homologous amino acids are found in regions that correspond to beta-strands in the crystal structures of TNF-alpha and LT-alpha [see, Eck and Sprang, *J. Biol. Chem.*, 264:17595-17605 (1989); Eck et al., *J. Biol. Chem.*, 267:2119-2122 (1992)].

EXAMPLE 2

Northern Blot Analysis

40 Expression of Apo-3 Ligand mRNA in human tissues and tumor cell lines was examined by Northern blot analysis (see Figure

4). Human RNA blots were hybridized to a  $^{32}\text{P}$ -labelled DNA probe generated by PCR using primers based on the sequence encoding an extracellular region of the Apo-3 Ligand polypeptide (amino acids 47 to 249 of Fig. 1):

- 5 CGACGACAAGCATATGCCGGCATCGCTGTCCGCCAGGAG (SEQ ID NO:5);  
CAGCCGGATCCTCGAGTCAGTGAACCTGGAAAGAGTCCG (SEQ ID NO:6). Human fetal,  
adult, or cancer cell line mRNA blots (Clontech) were incubated with  
the DNA probe in hybridization buffer (5X SSPE; 2X Denhardt's  
solution; 100 mg/mL denatured sheared salmon sperm DNA; 50%  
10 formamide; 2% SDS) for 60 hours at 42°C. The blots were washed  
several times in 2X SSC; 0.05% SDS for 1 hour at room temperature,  
followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The  
blots were developed after overnight exposure by phosphorimager  
analysis (Fuji).
- 15 As shown in Fig. 4, a single mRNA transcript of about 2  
kb was detected. This transcript was expressed in fetal kidney,  
liver, lung, and brain, and in many adult tissues, particularly in  
ovary, spleen and heart. Expression was also detected in the  
following human tumor cell lines: G361 melanoma, A549 lung carcinoma,  
20 SW480 colon carcinoma, and HeLa S3 cervical carcinoma.

#### EXAMPLE 3

##### Expression of Apo-3 Ligand in *E. coli*

- The DNA sequence (of Fig. 1; SEQ ID NO:2) encoding an  
25 extracellular region of the Apo-3 Ligand polypeptide (amino acids 47  
to 249 of Fig. 1; SEQ ID NO:1) was amplified with PCR primers (see  
Example 2 primers) and subcloned into the plasmid pET19B (Novagen)  
downstream and in frame of a Met Gly His<sub>10</sub> sequence followed by a 12  
amino acid enterokinase cleavage site (derived from the plasmid):  
30 Met Gly His His His His His His His His Ser Ser Gly His Ile  
Asp Asp Asp Asp Lys Met (SEQ ID NO: 7).

The resulting plasmid was used to transform *E. coli*  
strain JM109 (ATCC 53323) using the methods described in Sambrook et  
al., supra. Transformants were identified by PCR. Plasmid DNA was  
35 isolated and confirmed by restriction analysis and DNA sequencing.

Selected clones were grown overnight in liquid culture  
medium LB supplemented with antibiotics. The overnight culture was  
subsequently used to inoculate a larger scale culture. The cells  
were grown to a desired optical density, during which the expression  
40 promoter is turned on.

After culturing the cells for several more hours, the  
cells were harvested by centrifugation. The cell pellet obtained by

the centrifugation was solubilized using a microfluidizer in a buffer containing 0.1M Tris, 0.2M NaCl, 50 mM EDTA, pH 8.0. The solubilized Apo-3 Ligand protein was purified using Nickel-sepharose affinity chromatography.

5

EXAMPLE 4

Activation of NF- $\kappa$ B by Soluble Apo-3 Ligand

Several assays were conducted to determine whether soluble Apo-3 Ligand activates NF- $\kappa$ B.

10 In a first assay, HeLa cells (ATCC CCL 22) were treated with His-tagged soluble Apo-3 Ligand (described in Example 3) (10  $\mu$ g/ml) in duplicate or with vehicle for 30 minutes. His-tagged Apo-2 ligand (see WO 97/25428) or TNF-alpha (Pennica et al., Nature, 312:724-729 (1984)) were assayed for comparison. Nuclear extracts 15 were prepared and 1  $\mu$ g of nuclear protein was reacted with a  $^{32}$ P-labelled NF- $\kappa$ B-specific synthetic oligonucleotide probe ATCAGGGACTTCCGCTGGGGACTTCG (SEQ ID NO:8) [see, also, MacKay et al., J. Immunol., 153:5274-5284 (1994)].

20 The results are shown in Fig. 5A. As shown in Fig. 5A, the soluble Apo-3 Ligand polypeptide induced significant NF- $\kappa$ B activation in HeLa cells as measured by an electrophoretic mobility shift assay [Marsters et al., Proc. Natl. Acad. Sci., 92:5401-5405 (1995)]; the level of activation was comparable to activation observed for Apo-2 ligand, but weaker than activation by TNF-alpha.

25 In another assay, human 293 cells (ATCC CCL 1573) were treated as above (except the cells were incubated with the respective ligands for 3 hours) and NF- $\kappa$ B activation was determined in the electrophoretic mobility shift assay as above. The results are shown in Figure 5B.

30 The 293 cells were also tested in an assay in which the cells were transfected by calcium phosphate precipitation with empty vector (pRK5; EP 307,247) or with pRK5 expression plasmids encoding dominant-negative (DN) mutants of TRADD, RIP, TRAF2, or NIK (Tularik, South San Francisco, CA). After a 16 hour incubation, the cells were 35 treated with soluble Apo-3 Ligand or TNF-alpha and assayed for NF- $\kappa$ B activation as above. The results are shown in Figure 5C.

EXAMPLE 5

Chromosomal Localization of the Apo-3 Ligand gene

40 Chromosomal localization of the Apo-3 Ligand gene was

examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-3 Ligand cDNA:

- 5 CCGCAGTCGTCCCAGGCTGCCGGC (SEQ ID NO:9) and  
GGAGCTAGTGAGGTGGAGATGGG (SEQ ID NO:10) [Gelb et al., Hum. Genet.,  
98:141 (1996)]. Analysis of the PCR data using the Stanford Human  
Genome Center Database and the Whitehead Institute for Biomedical  
Research/MIT Center for Genome Research indicated that Apo-3 Ligand  
10 is linked to the marker SHGC-31370, with an LOD of 6.8, which maps to  
human chromosome 17p12-13. This analysis also showed that Apo-3  
Ligand is closely linked to the genomic locus of the p53 tumor  
suppressor.

EXAMPLE 6

- 15 Apoptotic Activity of Full length Apo-3 Ligand  
A pRK5 plasmid (see EP 307,247) encoding Apo-3 Ligand  
polypeptide (amino acids 1 to 249 of Fig. 1), or empty pRK5 plasmid,  
was transiently transfected along with a pRK5 plasmid encoding human  
CD4 as a marker for transfection into human HeLa cells by  
20 electroporation, and the cells were plated in tissue culture dishes.  
Four hours later, the caspase inhibitor z-VAD-fmk (Research  
Biochemicals) (200 µM) was added to some of the dishes. Twenty hours  
later, apoptosis was determined by FACS analysis of cells positive  
for the CD4 marker by measuring binding of fluorescein isothiocyanate  
25 (FITC)-conjugated annexin V (Brand Applications) (see WO 97/25428).

As shown in Figure 6, transfection by Apo-3 Ligand  
resulted in about a doubling of the level of apoptosis as compared  
with pRK5 (control). This increase in apoptosis was blocked by z-  
VAD-fmk, confirming the involvement of caspases in this effect.

30

EXAMPLE 7

- Activation of Jun N-terminal kinase by Full length Apo-3  
Ligand
- 35 The pRK5 plasmid encoding Apo-3 Ligand polypeptide or  
empty pRK5 plasmid (see Example 6 above) was transiently transfected  
into human 293 cells (ATCC CCL 1573) by calcium phosphate  
precipitation. Four hours later, one set of cells was treated with  
z-VAD-fmk (Research Biochemicals) (200 µM). Twenty hours later, the  
40 cells were harvested and analyzed for activity of Jun N-terminal  
kinase (JNK; also called stress activated protein kinase or SAPK)  
using a commercially available JNK/SAPK assay kit (New England

Biolabs), and according to the manufacturer's instructions.

As shown in Figure 7, transfection by Apo-3 Ligand resulted in a detectable increase in the level of JNK/SAPK activity as compared with the pRK5 control. Treatment with z-VAD-fmk 5 augmented JNK activation by Apo-3 Ligand, suggesting that JNK activation by Apo-3 Ligand is enhanced when apoptosis activation is prevented by z-VAD-fmk.

EXAMPLE 8

10 Binding of Apo-3 by Apo-3 Ligand

A binding assay was conducted to determine if Apo-3 Ligand binds Apo-3 receptor. Apo-3 receptor was described by Marsters et al., Curr. Biol., 6:750 (1996). Other investigators have also referred to Apo-3 as DR3 [Chinnayian et al., Science, 274:990 15 (1996)], Wsl-1 [Kitson et al., Nature, 384:372 (1996)], TRAMP [Bodmer et al., Immunity, 6:79 (1997)], or LARD [Screaton et al., PNAS, 94:4615-4619 (1997)].

A Flag-epitope tagged soluble Apo-3 was produced (as described for DR5 in Sheridan et al., Science, 277:818 (1997)) and 20 tested in a co-immunoprecipitation assay to examine its ability to associate with a histidine tagged soluble Apo-3 Ligand (see Example 3 above). For comparison, histidine-tagged Apo-2L [prepared as described in Pitti et al., J. Biol. Chem., 271:12687 (1996)] and the ECD of the Apo-2L receptor, DR5 [prepared as described in Sheridan et 25 al., Science, 277:818 (1997); see also, Pan et al., Science, 277:815 (1997)] were also tested in the assay. The respective ligands (1 µg/ml) and receptors (1 µg/ml) were incubated for 1 hour at room temperature. The reaction mixtures were subjected to precipitation with anti-Flag antibody conjugated to sepharose beads (Kodak) (Figure 30 8A, left) or nickel-sepharose (Qiagen) (Figure 8A, right) by overnight incubation at 4°C according to manufacturer's instructions and resolved by gel electrophoresis. The ligands were visualized by Western blot analysis with nickel-conjugated horseradish peroxidase. In Figure 8A, molecular weight markers (kDa) are indicated on the 35 left.

The data revealed that Apo-3 Ligand bound to Apo-3 but not to DR5, and that Apo-2 Ligand bound to DR5, but not to Apo-3. Thus, there appeared to be a specific complex formation between Apo-3 Ligand and Apo-3. Further, preincubation of ligand with Flag tagged 40 Apo-3 and immunodepletion of complexes with anti-Flag antibody-conjugated sepharose beads as above inhibited apoptosis induction by Apo-3 Ligand, but not by Apo-2L (see Figure 8B), supporting the

conclusion that Apo-3 Ligand binds to Apo-3.

EXAMPLE 9

Apoptotic Activity of Soluble Apo-3 Ligand

5 Human MCF-7 breast carcinoma cells (ATCC HTB 22) were pre-treated for 1 hour with cyclohexamide (CHX) (10 µg/ml) and incubated for 14 hours with soluble Apo-3 Ligand (2 µg/ml; prepared as described in Example 3 above) alone or together with zVAD-fmk (Research Biochemicals) (100 nM). The cells were then stained with  
10 Hoechst dye and photographed by phase (Figure 9A, left) and by fluorescence (Figure 9A, right) microscopy. In Figure 9A, arrowheads indicate some of the apoptotic cells (left) and their condensed nuclei (right).

15 The soluble Apo-3 Ligand induced marked apoptosis in the MCF-7 cells within a period of 14 hours (see Figure 9A) as evidenced by the morphological changes and chromatin condensation.

20 In a separate experiment, the MCF-7 cells were pre-treated for 1 hour with buffer or CHX, incubated for an additional 14 hours with the indicated concentrations of soluble Apo-3 Ligand (see Figure 9B), and apoptotic or live cells were score by microscopy. Apo-3 ligand-induced apoptosis was augmented by the translation inhibitor, cyclohexamide, indicating that this response is independent of de novo protein synthesis (see Figure 9B).

HeLa S3 cervical carcinoma cells (ATCC CCL 2.2) were  
25 similarly pre-treated with CHX and then incubated with soluble Apo-3 Ligand (prepared as described in Example 3 above) alone or together with zVAD-fmk or DEVD-fmk (Research Biochemicals) (100nM). The treated cells were then subjected to DNA fragmentation analysis. The soluble Apo-3 Ligand induced marked apoptosis in the HeLa S3 cells  
30 (see Figure 9C) as evidenced by internucleosomal DNA fragmentation. Addition of the caspase inhibitors, DEVD-fmk and zVAD-fmk, or transfection with the virus-derived caspase inhibitors CrmA or p35 (see below, Figure 9D) blocked induction of apoptosis by Apo-3 Ligand, indicating a requirement for caspase activity in this  
35 response.

MCF-7 cells were transfected by lipofection with empty vector (pRK5; EP 307,247) or with expression plasmid encoding the caspase inhibitors, p35 or CrmA, or a dominant-negative FADD mutant (Tularik, South San Francisco, CA). Sixteen hours later, the cells  
40 were pre-treated with CHX for 1 hour, and then treated for an additional 14 hours with soluble Apo-3 Ligand (2 µg/ml).

The results are shown in Figure 9D. Transfection with a dominant-negative mutant of FADD, which contains the adaptor's death domain [see, Hsu et al., Cell, 84:299 (1996)], prevented apoptosis induction by Apo-3 Ligand. Thus, Apo-3 Ligand appears to signal cell  
5 death through FADD itself or through a closely related protein.

EXAMPLE 10

Expression of Apo-3 Ligand in mammalian cells

This example illustrates preparation of Apo-3 Ligand by  
10 recombinant expression in mammalian cells.

The vector, pRK5 (see EP 307,247, published March 15,  
1989), is employed as the expression vector. Optionally, the Apo-3  
Ligand DNA is ligated into pRK5 with selected restriction enzymes to  
allow insertion of the Apo-3 Ligand DNA using ligation methods such  
15 as described in Sambrook et al., supra. The resulting vector is  
called pRK5-Apo-3 Ligand.

In one embodiment, the selected host cells may be 293  
cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in  
tissue culture plates in medium such as DMEM supplemented with fetal  
20 calf serum and optionally, nutrient components and/or antibiotics.

About 10 µg pRK5-Apo-3 Ligand DNA is mixed with about 1 µg DNA  
encoding the VA RNA gene [Thimmappaya et al., Cell, 31:543 (1982)]  
and dissolved in 500 µl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl<sub>2</sub>.

To this mixture is added, dropwise, 500 µl of 50 mM HEPES (pH 7.35),  
25 280 mM NaCl, 1.5 mM NaPO<sub>4</sub>, and a precipitate is allowed to form for  
10 minutes at 25°C. The precipitate is suspended and added to the  
293 cells and allowed to settle for about four hours at 37°C. The  
culture medium is aspirated off and 2 ml of 20% glycerol in PBS is  
added for 30 seconds. The 293 cells are then washed with serum free  
30 medium, fresh medium is added and the cells are incubated for about 5  
days.

Approximately 24 hours after the transfections, the  
culture medium is removed and replaced with culture medium (alone) or  
culture medium containing 200 µCi/ml <sup>35</sup>S-cysteine and 200 µCi/ml <sup>35</sup>S-  
35 methionine. After a 12 hour incubation, the conditioned medium is  
collected, concentrated on a spin filter, and loaded onto a 15% SDS  
gel. The processed gel may be dried and exposed to film for a  
selected period of time to reveal the presence of Apo-3 Ligand  
polypeptide. The cultures containing transfected cells may undergo  
40 further incubation (in serum free medium) and the medium is tested in  
selected bioassays.

In an alternative technique, Apo-3 Ligand may be introduced into 293 cells transiently using the dextran sulfate method described by Somparyrac et al., Proc. Natl. Acad. Sci., 78:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700 µg pRK5-Apo-3 Ligand DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5 µg/ml bovine insulin and 0.1 µg/ml bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed Apo-3 Ligand can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

In another embodiment, Apo-3 Ligand can be expressed in CHO cells. The pRK5-Apo-3 Ligand can be transfected into CHO cells using known reagents such as CaPO<sub>4</sub> or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as <sup>35</sup>S-methionine. After determining the presence of Apo-3 Ligand polypeptide, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the conditioned medium is harvested. The medium containing the expressed Apo-3 Ligand can then be concentrated and purified by any selected method.

Epitope-tagged Apo-3 Ligand may also be expressed in host CHO cells. The Apo-3 Ligand may be subcloned out of the pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into a Baculovirus expression vector. The poly-his tagged Apo-3 Ligand insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged Apo-3 Ligand can then be concentrated and purified by any selected method, such as by Ni<sup>2+</sup>-chelate affinity chromatography.

EXAMPLE 11Expression of Apo-3 Ligand in Yeast

The following method describes recombinant expression of Apo-3 Ligand in yeast.

5       First, yeast expression vectors are constructed for intracellular production or secretion of Apo-3 Ligand from the ADH2/GAPDH promoter. DNA encoding Apo-3 Ligand, a selected signal peptide and the promoter is inserted into suitable restriction enzyme sites in the selected plasmid to direct intracellular expression of  
10 Apo-3 Ligand. For secretion, DNA encoding Apo-3 Ligand can be cloned into the selected plasmid, together with DNA encoding the ADH2/GAPDH promoter, the yeast alpha-factor secretory signal/leader sequence, and linker sequences (if needed) for expression of Apo-3 Ligand.

15      Yeast cells, such as yeast strain AB110, can then be transformed with the expression plasmids described above and cultured in selected fermentation media. The transformed yeast supernatants can be analyzed by precipitation with 10% trichloroacetic acid and separation by SDS-PAGE, followed by staining of the gels with Coomassie Blue stain.

20      Recombinant Apo-3 Ligand can subsequently be isolated and purified by removing the yeast cells from the fermentation medium by centrifugation and then concentrating the medium using selected cartridge filters. The concentrate containing Apo-3 Ligand may further be purified using selected column chromatography resins.

25

EXAMPLE 12Expression of Apo-3 Ligand in Baculovirus

The following method describes recombinant expression of Apo-3 Ligand in Baculovirus.

30      The Apo-3 Ligand is fused upstream of an epitope tag contained with a baculovirus expression vector. Such epitope tags include poly-his tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393

35      (Novagen). Briefly, the Apo-3 Ligand or the desired portion of the Apo-3 Ligand (such as a sequence encoding an extracellular domain, e.g., amino acids 47 to 249 of Fig. 1 (SEQ ID NO:1)) is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking (selected) restriction enzyme sites.

40      The product is then digested with those selected restriction enzymes and subcloned into the expression vector.

Recombinant baculovirus is generated by co-transfected

the above plasmid and BaculoGold™ virus DNA (Pharmingen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses are harvested and used for  
5 further amplifications. Viral infection and protein expression is performed as described by O'Reilley et al., *Baculovirus expression vectors: A laboratory Manual*, Oxford:Oxford University Press (1994).

Expressed poly-his tagged Apo-3 Ligand can then be purified, for example, by Ni<sup>2+</sup>-chelate affinity chromatography as  
10 follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert et al., *Nature*, 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM MgCl<sub>2</sub>; 0.1 mM EDTA; 10% Glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The  
15 sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% Glycerol, pH 7.8) and filtered through a 0.45 µm filter. A Ni<sup>2+</sup>-NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated  
20 with 25 mL of loading buffer. The filtered cell extract is loaded onto the column at 0.5 mL per minute. The column is washed to baseline A<sub>280</sub> with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% Glycerol, pH 6.0), which elutes  
25 nonspecifically bound protein. After reaching A<sub>280</sub> baseline again, the column is developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by SDS-PAGE and silver staining or western blot with Ni<sup>2+</sup>-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing  
30 the eluted His<sub>10</sub>-tagged Apo-3 Ligand are pooled and dialyzed against loading buffer.

Alternatively, purification of the IgG tagged (or Fc tagged) Apo-3 Ligand can be performed using known chromatography techniques, including for instance, Protein A or protein G column chromatography.  
35

#### EXAMPLE 13

##### Preparation of Antibodies that Bind Apo-3 Ligand

This example illustrates preparation of monoclonal  
40 antibodies which can specifically bind Apo-3 Ligand.

Techniques for producing the monoclonal antibodies are known in the art and are described, for instance, in Goding, supra.

Immunogens that may be employed include purified Apo-3 Ligand, fusion proteins containing Apo-3 Ligand, and cells expressing recombinant Apo-3 Ligand on the cell surface. Selection of the immunogen can be made by the skilled artisan without undue experimentation.

5 Mice, such as Balb/c, are immunized with the Apo-3 Ligand immunogen emulsified in complete Freund's adjuvant and injected subcutaneously or intraperitoneally in an amount from 1-100 micrograms. Alternatively, the immunogen is emulsified in MPL-TDM adjuvant (Ribi Immunochemical Research, Hamilton, MT) and injected  
10 into the animal's hind foot pads. The immunized mice are then boosted 10 to 12 days later with additional immunogen emulsified in the selected adjuvant. Thereafter, for several weeks, the mice may also be boosted with additional immunization injections. Serum samples may be periodically obtained from the mice by retro-orbital  
15 bleeding for testing in ELISA assays to detect Apo-3 Ligand antibodies.

After a suitable antibody titer has been detected, the animals "positive" for antibodies can be injected with a final intravenous injection of Apo-3 Ligand. Three to four days later, the  
20 mice are sacrificed and the spleen cells are harvested. The spleen cells are then fused (using 35% polyethylene glycol) to a selected murine myeloma cell line such as P3X63AgU.1, available from ATCC, No. CRL 1597. The fusions generate hybridoma cells which can then be plated in 96 well tissue culture plates containing HAT (hypoxanthine,  
25 aminopterin, and thymidine) medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells will be screened in an ELISA for reactivity against Apo-3 Ligand. Determination of "positive" hybridoma cells secreting the desired monoclonal antibodies against  
30 Apo-3 Ligand is within the skill in the art.

The positive hybridoma cells can be injected intraperitoneally into syngeneic Balb/c mice to produce ascites containing the anti-Apo-3 Ligand monoclonal antibodies. Alternatively, the hybridoma cells can be grown in tissue culture  
35 flasks or roller bottles. Purification of the monoclonal antibodies produced in the ascites can be accomplished using ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can be employed.

EXAMPLE 14Use of Apo-3 Ligand as a hybridization probe

The following method describes use of a nucleotide sequence encoding Apo-3 Ligand as a hybridization probe.

- 5       DNA comprising the coding sequence of Apo-3 Ligand (as shown in Figure 1, SEQ ID NO:2) is employed as a probe to screen for homologous DNAs (such as those encoding naturally-occurring variants of Apo-3 Ligand) in human tissue cDNA libraries or human tissue genomic libraries.
- 10      Hybridization and washing of filters containing either library DNAs is performed under the following high stringency conditions. Hybridization of radiolabeled Apo-3 Ligand-derived probe to the filters is performed in a solution of 50% formamide, 5x SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 50 mM sodium phosphate, pH 6.8, 15 2x Denhardt's solution, and 10% dextran sulfate at 42°C for 20 hours. Washing of the filters is performed in an aqueous solution of 0.1x SSC and 0.1% SDS at 42°C.
- 15      DNAs having a desired sequence identity with the DNA encoding full-length native sequence Apo-3 Ligand can then be identified using standard techniques known in the art.

\* \* \* \*

Deposit of Material

- The following materials have been deposited with the  
25 American Type Culture Collection, 10801 University Boulevard,  
Manassas, Virginia, USA (ATCC):  

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
DNA30879-1152	209358	October 10, 1997

30

- This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of  
35 Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between  
40 Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one

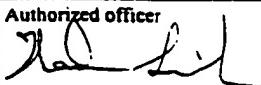
determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

5           The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a  
10          license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

15          The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute  
20          an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in  
25          addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page _____, line _____	
B. IDENTIFICATION OF DEPOSIT	
Name of depositary institution <b>AMERICAN TYPE CULTURE COLLECTION</b>	
Address of depositary institution ( <i>including postal code and country</i> ) <b>10801 University Boulevard Manassas, Virginia USA</b>	
Date of deposit <b>October 10, 1997</b>	Accession Number <b>209358</b>
C. ADDITIONAL INDICATIONS ( <i>leave blank if not applicable</i> )	
This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ( <i>if the indications are not for all designated States</i> )	
E. SEPARATE FURNISHING OF INDICATIONS ( <i>leave blank if not applicable</i> )	
The indications listed below will be submitted to the International Bureau later ( <i>specify the general nature of the indications e.g., "Accession Number of Deposit"</i> )	
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Authorized officer 	
For International Bureau use only	
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Authorized officer	

What is claimed is:

1. An isolated Apo-3 Ligand polypeptide having at least about 80% amino acid sequence identity with native sequence Apo-3 Ligand polypeptide comprising amino acid residues 1 to 249 of Fig. 1 (SEQ ID NO:1).
2. The Apo-3 Ligand polypeptide of claim 1 wherein said Apo-3 Ligand polypeptide has at least about 90% amino acid sequence identity.
- 10 3. The Apo-3 Ligand polypeptide of claim 2 wherein said Apo-3 Ligand polypeptide has at least about 95% amino acid sequence identity.
- 15 4. The Apo-3 Ligand polypeptide of claim 1 wherein said Apo-3 Ligand polypeptide binds to Apo-3 receptor.
5. An isolated native sequence Apo-3 Ligand polypeptide comprising amino acid residues 1 to 249 of Fig. 1 (SEQ ID NO:1).
- 20 6. An isolated Apo-3 Ligand polypeptide comprising amino acid residues 47 to 249 of Fig. 1 (SEQ ID NO:1).
7. An isolated Apo-3 Ligand polypeptide comprising amino acid residues X to 249, wherein X is any one of amino acid residues 42 to 25 52 of Figure 1 (SEQ ID NO:1).
8. An isolated tumor necrosis factor (TNF) homolog comprising a Type II transmembrane polypeptide which binds Apo-3 receptor.
- 30 9. A chimeric molecule comprising the Apo-3 Ligand polypeptide of claim 1 or the sequence of claim 6 fused to a heterologous amino acid sequence.
- 35 10. The chimeric molecule of claim 9 wherein said heterologous amino acid sequence is an epitope tag sequence.
11. The chimeric molecule of claim 9 wherein said heterologous amino acid sequence is an immunoglobulin sequence.
- 40 12. The chimeric molecule of claim 11 wherein said immunoglobulin sequence is an IgG Fc domain.

13. An antibody which binds to the Apo-3 Ligand polypeptide of claim 1 or the sequence of claim 6.
- 5 14. The antibody of claim 13 wherein said antibody is a monoclonal antibody.
15. The antibody of claim 13 which comprises a chimeric antibody.
- 10 16. The antibody of claim 13 which comprises a human antibody.
17. An isolated nucleic acid comprising DNA encoding Apo-3 Ligand polypeptide having amino acid residues 47 to 249 of Fig. 1 (SEQ ID NO:1).
- 15 18. The nucleic acid of claim 17 comprising DNA encoding Apo-3 Ligand polypeptide having amino acid residues 1 to 249 of Fig. 1 (SEQ ID NO:1).
- 20 19. A vector comprising the nucleic acid of claim 17.
20. The vector of claim 19 operably linked to control sequences recognized by a host cell transformed with the vector.
- 25 21. A host cell comprising the vector of claim 19.
22. The host cell of claim 21 wherein said cell is a CHO cell.
23. The host cell of claim 21 wherein said cell is an *E. coli*.
- 30 24. The host cell of claim 21 wherein said cell is a yeast cell.
25. A process for producing Apo-3 Ligand polypeptides comprising culturing the host cell of claim 21 under conditions suitable for expression of Apo-3 Ligand and recovering Apo-3 Ligand from the cell culture.
- 35 26. An isolated Apo-3 Ligand polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209358.
- 40 27. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cancer cells to an effective amount of

Apo-3 Ligand polypeptide.

28. A method of inducing NF- $\kappa$ B-dependent gene transcription in mammalian cells comprising exposing mammalian cells to an effective amount of Apo-3 Ligand polypeptide.

29. A method of inducing JNK/SAPK-dependent responses in mammalian cells comprising exposing mammalian cells to an effective amount of Apo-3 Ligand polypeptide.

4

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1401 AAGGGGGCC GCGACTCTAG A  
TTCCCCCGG CGCTGAGATC T

Fig. 1 (cont.)

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ACATACTTTGGGCTTTAAAGTTGAGATATAGATTGAGCCCCAGTTTGGAGTGTTATCTTATTTCTGGATGTTGG  
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CTCAGTATCCATCC

Fig. 2

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 NLTD    1 M G A L C L E G A G G G R I O C R S I L L A V A C A T S L V I L L L A V P I T V L A V L A L V P O D  
 HCDOL    1 - - M I E T Y N O T S P R S A A T C L P I S M K I F M Y L L I V F L I I O N I G S A L F A V Y L H R

DNA 30879    17 R - A S L S A D E P A D E E I L V - - A E E D D O P S E L N P O T E E S O D P A P F L N R L V R P R  
 NLTD    51 O C G L V T E T A D P G A O A D O G L G F D K L P E E E P E T O L S P G L P A A H L I G A P L N G O  
 HCDOL    49 R L D K I E D E R N L H E D F V F U K T I O R C H T G E R S L S L L N C E E I K S O F E G F V K D I

DNA 30879    93 R S A P K G R K T R A R R A I A A H T E V H P R P C Q D G A D A G V D G I . V S C M . E E A R I N S  
 NLTD    101 G C G W E T T K E O A F - - L T S C I O F S D A E Q I A L P D G L - - - V V L Y C L V G Y R G R  
 HCDOL    99 M W N K E E T K K E N S F E M O K G D O H P O I A A H V I S E A S S K I T S V L O M A E K C Y Y I M

DNA 30879    142 S S P L R Y N R O I G E F I V R A G L Y Y L Y C O V M F - - - D E C K A V Y L - K L O L L V D  
 NLTD    145 A P P G G G D P O Q R S V I L - R S S I Y - R A G G A Y - - - G P O T P - - - E L L E  
 HCDOL    149 S M N L V T L E N G K O L I V K R O G L Y Y I Y A O Y T P C S N R E A S S O A P F I A S L C I K S P

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 HCDOL    169 G R F E . R I A L R A A N H S S A < P C G O O S I H L G O V F E L O P C A S V F V N V T D P S O V

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 NLTD    226 D F A A R O K T I F F G A V M V D  
 HCDOL    228 S H G T G F T S F G L L K L

Fig. 3A

Fig. 36

		A	B'		
Apo3L	99	RKTRAFRAIAAHYEVHPRP-GQ-----DGAQAGVDTGTVSGWEARIN--SSSPRLRYNRQIG			
TNF	82	R-TPSDK-PVAHVANPQAEGQ-----LQ-----WLNRRANA-LLANGVELR-DN			
CD40L	114	QKGDQNPQIAAHVISEASS-----KTTSVLQ-----WAEKGYYT-MSNNLVTLENGK			
LTb	80	ETDLSPGLPAAHILIGAPLK-GQ-----GLG-----WETTKEQA-FLTSGTQFSDAE			
Apo2L	115	RERGPQR-VAAHITGTRGRSNTLSSPNSKNEKAIGRKINSWESSRSGHSFLSNLH-LR-NG			
CD95L	138	PEKKELR-KVAELTGKSNS-----RSMPLE-----WEDTYGIV-LLSG-VKYK-KG			
Lta	56	LAHSTLK-PAAHLIGDPSK-----QNSLL-----WRANTDRA-FLQDGFSLS-NN			
		B	C	D	E
Apo3L	152	EIVTRA-GLYVHLYCQVHDE-G-----KAVYLKLDLLVDGV-----LALRCLEEPSATA			
TNF	123	QLVVPSEGLYLIVSOVLFKGQGCP-----THVLLHTHSRIAVSY---QTKVNLLSAIKSP			
CD40L	144	QLTVKROGLYIIVAQVTFCSNREASSQAPPFIASLCLKSPG-----RFERILLRAANTH			
LTb	123	GLALPODGIRVYCLVGYRGRAPPGGDPQGRSVTLRSSLYRAGGAYCPGTPELLLEGAET			
Apo2L	173	ELVIHEKGFYIIVSQTYFRPOEEIKENTKNDKQMVQYIYKYS-T-Y---PD-PILLMKSARN			
CD95L	180	GLVINEIGLAFVVISKVYFRGGSC-----NNLPESHKVYMRNSKYP--QDLVMMEGKMMSY			
Lta	98	SLLVPTSGIIFVVISQVVFSGKAYSPKATSSPLYLAHEVQLFSSQY---PFHVPLLSSQKMV			
		F	G	H	
Apo3L	199	-----ASSLGPOLRLCQVSGLLAL--RPGSSLRIRTL-PWAHLKAAPPLTYFGLFQVH			
LTb	184	VTPVLDPARQGYGPLWYTSVGFQGLVQLRRGERVYVNISHPDMVDFARGK-TFFGAVMVG			
CD40L	213	-----SSAKPCGQQSIHLGGVFELOPGASVFNVTDPQSQVSHGTGF-TSFGLLKL			
TNF	178	CQRETPEGA-EAKPWYEPIYLGGVFQLEKGDRLSAEIN--RPDYLDFAESGQVYFGIIAL			
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CD95L	234	-----CTTGQMWARSSYLGAVFNLTSADHLYVNVSSEL--SLVNFEESQ-TFFGLYKL			
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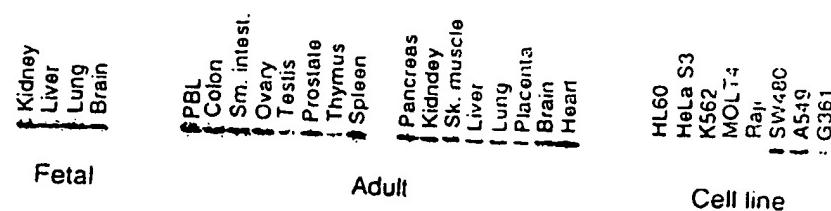


Fig. 4

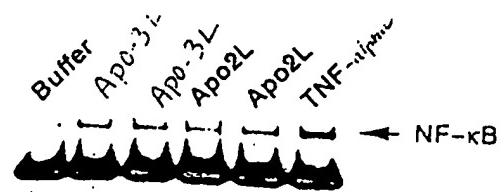


Fig. 5

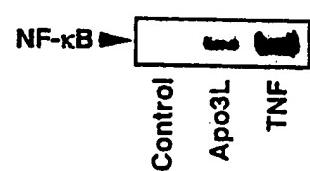
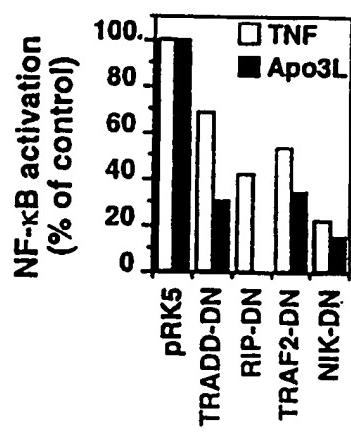


Fig. 5B

Fig.  
5C

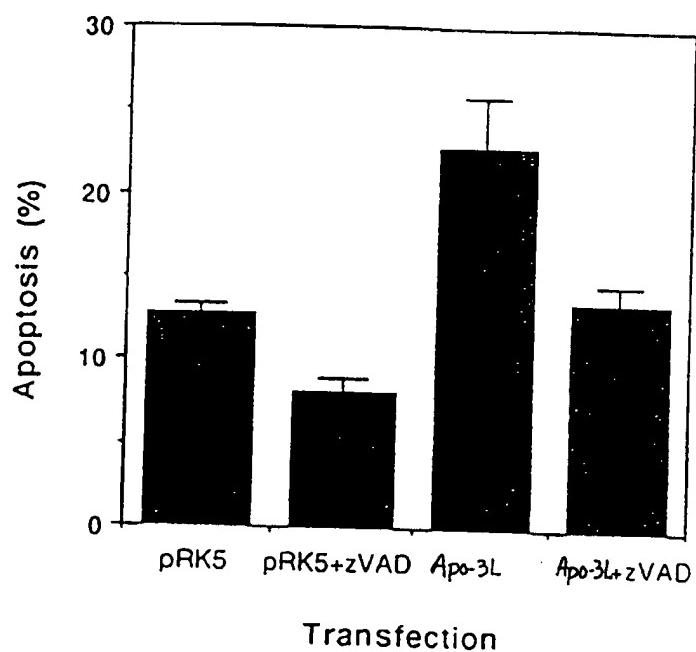


Fig. 6

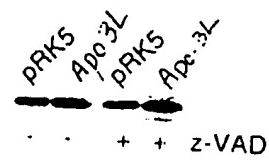


Fig. 7

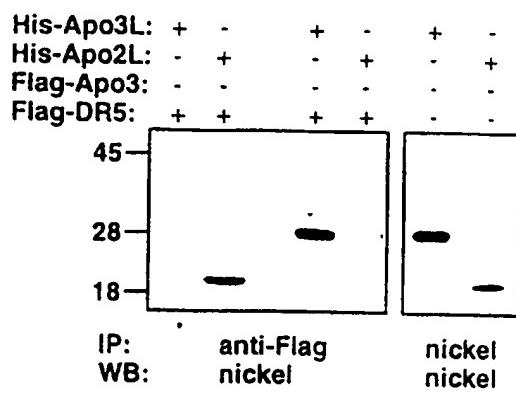
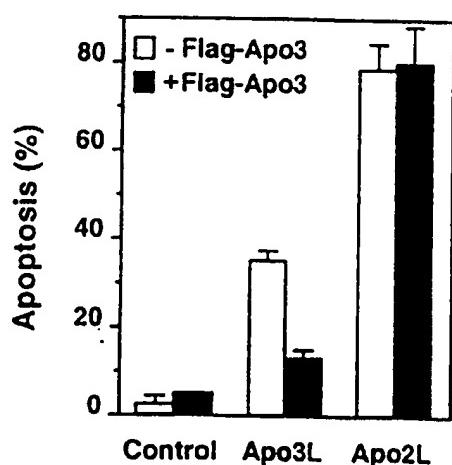
**A**

Fig. 8

**B**

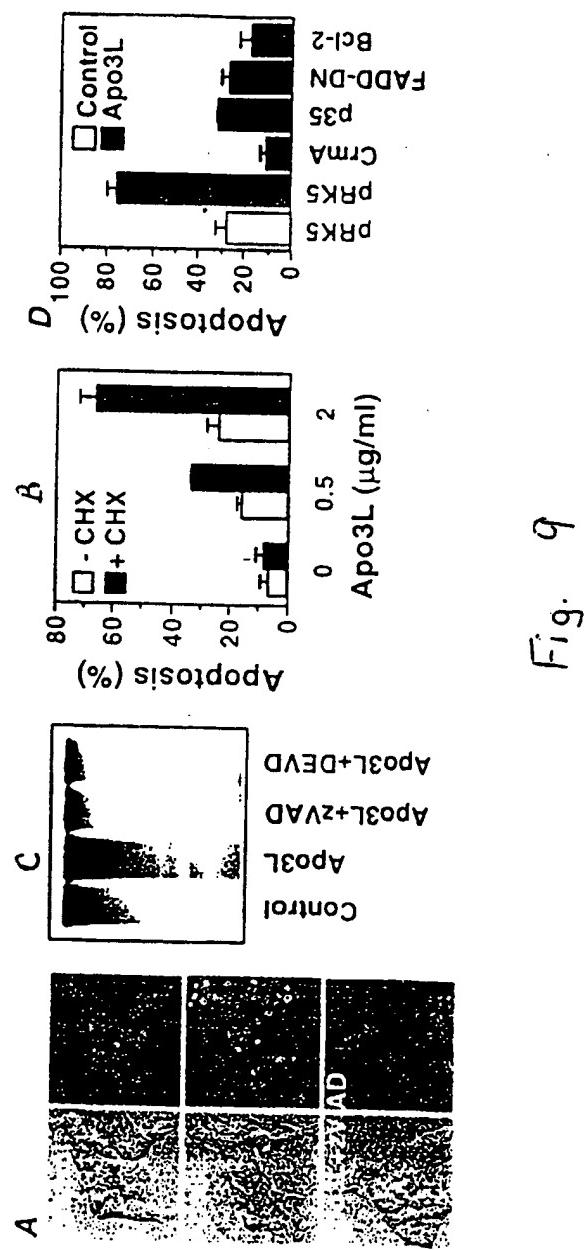


Fig. 9

## SEQUENCE LISTING

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<141> 1998-10-09

<150> 60/062,037

<151> 1997-10-10

<150> 60/069,862

<151> 1997-12-17

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<170> PatentIn Ver. 2.0

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Val His Pro Arg Pro Gly Gln Asp Gly Ala Gln Ala Gly Val Asp Gly  
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Thr Val Ser Gly Trp Glu Glu Ala Arg Ile Asn Ser Ser Ser Pro Leu  
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# INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 98/21407

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C12N15/19 C07K14/705 C12N15/62 C07K16/24 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 37020 A (GENENTECH INC) 9 October 1997 see the whole document ---	
A	WO 97 25428 A (GENENTECH INC) 17 July 1997 see the whole document ---	
A	ASHKENAZI, A. ET AL.: "Induction of apoptosis by Apo-2 ligand, a new member of the TNF cytokine family." FASEB JOURNAL, vol. 10, no. 6, 30 April 1996, pages a1276-aBSTR.1600, XP002092398 see abstract --- -/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents such combination being obvious to a person skilled in the art

"S" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

5 February 1999

18/02/1999

Name and mailing address of the ISA

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Smalt, R

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/21407

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 05783 A (CHICHEPORTICHE YVES ; FACULTY OF MEDICINE OF THE UNI (CH); BIOGEN I) 12 February 1998 see page 3, paragraph 3 see page 31, paragraph C see page 35, line 5 - line 22 ----	1-10, 13-21, 25-27
P,X	CHICHEPORTICHE ET AL: "TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 51, 19 December 1997, pages 32401-10, XP002076164 see page 32403, right-hand column, paragraph 2; figures 1,2 see page 32407, right-hand column, line 16 - line 18 ----	1-8,13, 17-21, 25-27
P,X	WO 98 35061 A (ABBOTT LAB) 13 August 1998  see page 13, paragraph 2 - page 14, paragraph 1 see page 66, paragraph 2 see page 71, paragraph 2 - paragraph 4 see page 77, paragraph 3 - page 79, paragraph 2; examples 10,11,13,14 ----	1-10,13, 14,16-26
P,X	MARSTERS, S.A. ET AL.: "Identification of a ligand for the death-domain-containing receptor Apo3." CURRENT BIOLOGY, vol. 8, no. 9, 23 April 1998, pages 525-8, XP002092399 see the whole document -----	1-10, 17-21, 23,25-28

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/21407

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 27-29, in as far as they relate to in vivo use, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2.  Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3.  Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/US 98/21407

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9737020 A	09-10-1997	AU	2598697 A	22-10-1997
WO 9725428 A	17-07-1997	AU CA EP	1824897 A 2241572 A 0873407 A	01-08-1997 17-07-1997 28-10-1998
WO 9805783 A	12-02-1998	AU	3829497 A	25-02-1998
WO 9835061 A	13-08-1998		NONE	